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RESEARCH AND TECHNOLOGY BRANCH

BACKGROUND INFORMATION TO SUPPORT THE POTENTIAL USE OF STREPTOCOCCUS FAECIUM SUBSP. CASSELIFLAVUS AND BIFIDOBACTERIUM SPP. AS INDICATORS OF HUMAN AND/OR ANIMAL SOURCES OF POLLUTION

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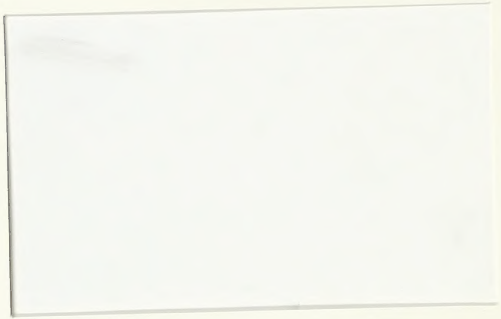

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PUBLICATIONS RELATED TO THIS PROJECT

Seyfried, P.L., T. Bleier, and M. Young. 1990. Bacterial indicators for the detection of sanitary waste in storm sewers. Proceedings Second Biennial Water Quality Symposium Microbiological Aspects, pp. 121-128.

ABSTRACT

Earlier studies (Seyfried et al., 1990) comparing the bacteria present in storm and sanitary sewers showed that higher concentrations of Streptococcus faecium subsp. casseliflavus could be found in non-priority storm sewer water and in storm water runoff. The microbial pollution in these waters was from animal sources. In comparison, sanitary sewage with human pollution input contained very low levels of the organism.

Levels of bifidobacteria were higher in sanitary and high priority storm sewage than in non-priority storm water suggesting that these bacteria may be indicators of recent human pollution.

Because Bifidobacterium species and S. faecium subsp. casselifalvus have not thus far been recognized as water quality indicators, a literature review was undertaken to elucidate the properties of these organisms. A summary of the review is provided.

Streptococcus faecium subsp. casseliflavius is distinct from the other enterococci because it is pigmented and because of the high pH at which growth is initiated and terminated. The organism also ferments raffinose and xylose. DNA homology studies showed that S. faecium subsp. casseliflavus is not genetically related to S. faecium or S. faecalis. Although mE agar and mEnterococcus agar are used for the isolation of enterococci and fecal streptococci, as yet no specific selective medium has been developed for S. faecium subsp. casseliflavus.

Survival studies of fecal streptococci in water, soil, and frozen foods showed that S. faecalis and S. faecium could survive for longer periods at cold temperatures than E. coli.

Although studies indicate that streptococcal levels may reach 10^5 to 10^9

per gram of human feces there have been no reports in the literature of S. faecium subsp. casseliflavus being part of the human gut flora. Streptococcus faecalis and S. faecium can be isolated in large numbers from the feces of both adults and children; S. bovis and S. avium are predominant in the feces of children as well.

It has been suggested that, while typical S. faecalis may not always be the predominant type of Streptococcus in human feces, it is not usually found in high levels in the feces of domestic animals. Thus, its presence in water could indicate contamination of human origin. Other studies have shown that S. bovis is a predominant species in cattle and sheep.

The streptococcal flora of wild animals appears to vary with the season and the geographic location as well as with diet, age, and host species. Unlike the domestic animal survey results, S. faecalis was isolated most frequently from all wild mammals except the wild boar.

The incidence of fecal streptococci on plants is important for the following reasons. First, streptococci on plants may be introduced into soil or water; and second, recovery in water of fecal streptococci that originated on plants would lead to erroneous fecal pollution indicator data. Research has shown that enterococci may be temporary residents on plants; they can be disseminated among plants by the action of insects and wind. Enterococci are apparently not native to soil; their isolation from soil samples can be linked to contamination from either plant or animal sources.

To be a good indicator of fecal pollution, organisms must be present in high enough levels to permit detection, be easy to enumerate, have survival characteristics and population densities similar to those of pathogens, be unable to multiply outside the human or animal intestine, and be released into the

environment only in their feces. Our previous studies (Seyfried et al., 1990) have shown that many of the properties of S. faecium subsp. casseliflavus are consistent with these guidelines. Additional work is needed to assess the impact of vegetation and to improve the methods for recovery of S. faecium subsp. casseliflavus.

Another genus that is under consideration as a water quality indicator is Bifidobacterium. Bifidobacteria are anaerobic, gram-positive pleomorphic rods found in the digestive tracts of higher animals. There are 24 species currently listed in Bergey's Manual of Determinative Bacteriology (Scardovi, 1986).

The organisms are characterized by their method of degrading glucose anaerobically to acetic and lactic acids by means of a fructose-6-phosphate shunt pathway. In addition, the enzymes glucosidase and α-galactosidase are present in almost all bifidobacteria.

A number of different selective media have been described for the recovery of Bifidobacterium spp. One of the major isolation problems is contamination of the media by fecal streptococci. A resuscitation procedure using an enrichment medium with incubation anaerobically for six hours at 37°C may be helpful in recovering bifidobacteria from environmental samples.

Survival studies carried out with B. adolescentis showed that, in waters with high nutrient levels, the organism had a higher rate of survival than E. coli. However, E. coli was able to survive longer than B. breve at room temperature in Lake Ontario water. It is expected that once outside their natural habitat, the bifidobacteria will not remain viable for prolonged periods of time.

Bifidobacteria are thought to be beneficial to their host and numerous articles have been written concerning their incidence in humans. Research has

shown that rapid colonization of the intestinal tract occurs after birth; bifidobacterial levels remain high in adults but often decrease in older people. According to Bergey's Manual, seven of the 24 Bifidobacterium species (i.e. B. bifidium, B. longum, B. breve, B. adolescentis, B. angulatum, B. catenulatum, and B. dentium) are found in human adult feces.

Animal studies revealed that lactobacilli and bifidobacteria numbers varied with the species of animal. It was found that, in general, animals of the same species had a common pattern of fecal flora but the patterns differed from those of other species.

There have been only a limited number of studies on the species and densities of bifidobacteria in sewage, water and sediment. Most authors found that bifidobacteria and E. coli counts from these sources were comparable. Water samples collected close to a river bottom were shown to contain B. animalis, B. breve, and B. longum species.

The fact that bifidobacteria are present in feces and appear to die off rapidly in the environment suggests that they are potentially good indicators of recent fecal pollution. However, further work must be done to improve the media for their isolation and to enhance their recovery from environmental samples.

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Taxonomy and Nomenclature

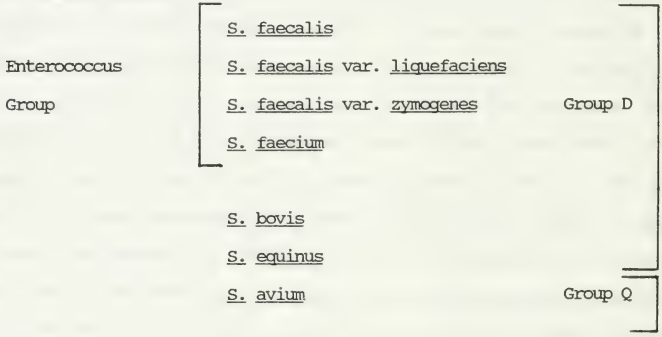
Streptococcal species are divided into several groups according to their ecological niches (Mundt, 1982). The lactic acid group are found only in the plant and dairy environment. Members of the pyogenic group are associated only with homeothermic hosts. The virulent pyogenic group, including Streptococcus pyogenes and S. equi are restricted to single hosts - the human and the horse- whereas others, such as S. agalactiae can be found in several hosts. The intestinal dwellers of the subthermophilic group, S. bovis and S. equinus, occur in several animal species and are not isolated from other sources. S. faecalis of the "enteric" group may actually consist of two groups: one restricted to the human host and a second distributed among wild animals, plants, and insects. The second group differs from the human by the ability to hydrolyze starch and to produce a rennin - proteolytic type of curd in milk. Although S. faecium is widely distributed in both the homeothermic and poikilothermic environments, no properties which differentiate the sources are known. S. faecium subsp. casseliflavus has been isolated from insects and plants, whereas S. uberis occurs on the bovine lips and skin. Many unidentified streptococci resembling S. lactis, S. faecalis and S. faecium can be isolated from all environments and vary widely in their properties.

According to the 16th edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1985) "Fecal Streptococcus" and "Lancefield's Group D Streptococcus" are terms that have been used synonymously. The following species are implied when this group is used as an indicator of fecal contamination: S. faecalis, S. faecalis var. liquefaciens, S. faecalis var. zymogenes, S. faecium, S. bovis, and S. aquinus.

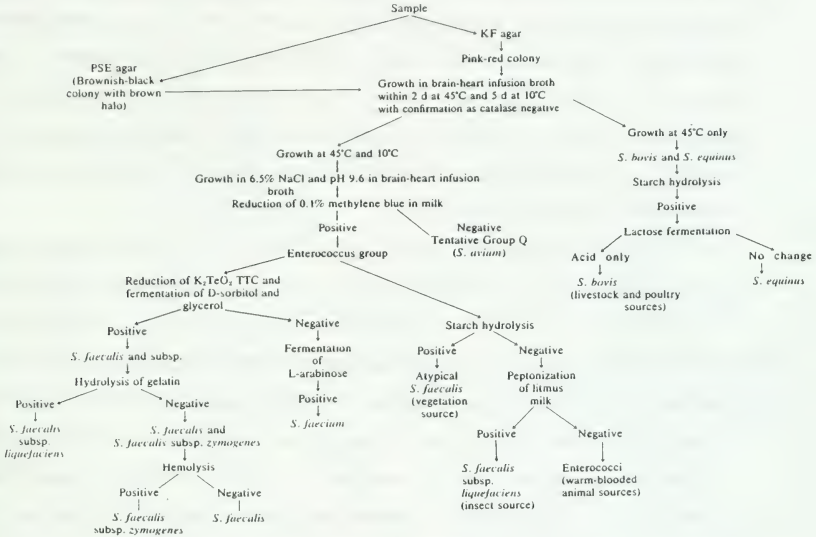
S. avium is the characteristic species of streptococci belonging to Lancefield's serological Group Q. It is isolated frequently from the feces of chickens and occasionally from the feces of humans, dogs, and pigs.

The term "enterococcus group" excludes *S. bovis*, *S. equinus*, and Group Q organisms. As a result, the restrictive media that are specific for the enterococcus group may not indicate the full extent of contamination by streptococci from fecal sources.

The subgroups within the fecal streptococcus group are indicated in the following diagram.



A schematic outline for the identification of fecal streptococci is provided:



In Volume 2 of Bergey's Manual (Sneath et al., 1986) the proposal of Schleifer and Kilpper-Balz for the creation of a new genus, Enterococcus, to encompass the enterococcal group, such as S. faecalis and S. faecium is noted. The formation of this genus, originally suggested some years earlier by Kalina (1970), is now fully supported by data from DNA/DNA and DNA/r RNA hybridization studies (Kilpper-Balz et al., 1982; Schleifer and Kilpper-Balz, 1984). Additional work by Collins et al. (1984) has led to the suggestion that other streptococcal species be transferred to the genus Enterococcus as E. avium, E. casseliflavus, E. durans, E. gallinarum and E. malodoratus.

The 17th edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1989) has added S. gallinarum to the Group D fecal streptococci.

The book states that although S. faecalis subsp. liquefaciens and S. faecalis subsp. zymogenes are differentiated based upon their ability to liquify gelatin and hemolyze red cells, the validity of these subspecies is questionable (Jacob et al., 1973; Oliver et al., 1977). Some streptococcal species such as S. bovis, S. equinus, and S. avium predominate in some animal species and not in others, but one cannot differentiate the source of fecal contamination based on fecal streptococcus speciation.

Previous editions (for example the 16th edition) of Standard Methods have suggested that the ratio of fecal coliforms to fecal streptococci could be used to indicate the source of contamination. However, the reliability of this ratio has been questioned because of variable survival rates of fecal streptococcus species. S. bovis and S. equinus tend to die off rapidly once they are exposed to aquatic environments, whereas S. faecalis and S. faecium will survive longer. Disinfection of wastewater, with chlorine for example, has a significant effect on the ratio of these indicators (Rosser and Sartory, 1982). Different methods of enumerating fecal streptococci will also affect the ratio. For example, the KF membrane filter procedure has a false-positive rate ranging from 10 to 90 percent in fresh and marine waters (Fujioka et al., 1984; Olivieri et al., 1977; Ericksen et al., 1983). For these reasons the APHA (1989) suggested that the FC/FS ratio should not be used as a method of differentiating human and animal sources of pollution.

Standard Methods, 17th Edition, describes the enterococcus group as streptococci having the ability to grow in 6.5% sodium chloride, at pH 9.6, and at 10°C and 45°C. The group includes S. faecalis, S. faecium, S. avium, and S. gallinarum. A comparison of other biochemical reactions for these organisms is provided in the table below.

SELECTED KEY BIOCHEMICAL CHARACTERISTICS OF THE STREPTOCOCCUS SPECIES WITHIN THE FECAL STREPTOCOCCUS AND ENTEROCOCCUS GROUPS[a]

Test	Fecal Streptococcus Group					
	Enterococcus Group					
	S. faecalis	*S. faecium*	*S. avium*	*S. gallinarium*	*S. bovis*	*S. equinus*
Catalase	−	−	−	−	−	−
40% Bile	+	+	+	+	+	+
Esculin[b]	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	−	−
Growth at 10°C	+	+	+	+	−	−
Pyruvate utilization[7]	+	−	−	−	−	−
Phosphatase activity[8]	+	−	+	+	−	−
Arginine hydrolysis[8]	+	+	−[d]	−	−	−
L-Sorbose fermentation[9]	−	−	+	−	−	−
Lactose fermentation[8]	+	+	+	+	+	+
n-acetyl-β-glucoseaminidase activity[9]	−[d]	−	−	+	−	−
Starch[8]	−	−	−	−	+	−
Arabinose[6]	−	+	+	−	−	−

[a] + = 90% or more of strains are positive
− = 90% or more of strains are negative
[d] = reactions variable

Streptococcus faecium var. casseliflavus was first described by Mundt and Graham in 1968. The properties which they described for the organism are given in the Table below.

Table 1. Properties of Streptococcus faecium var. casseliflavus

Property	No. of cultures	Percentage
Growth in broth		
At 10°C	361	99.8
At 45°C	361	68.7
At pH 9.6	361	96.9
With 6.5% NaCl	361	98.3
Growth on agar		
With 5% bile salt	361	99.1
With potassium tellurite	361	96.0
Reduction of		
Litmus	361	100.0
Methylene blue	361	100.0
Triphenyltetrazolium-HCl	361	100.0
Deamination of arginine	361	14.0
Dissimilation of malate	50	100.0
Production of peroxidase	50	100.0
Hydrolysis of hippurate	50	84.0
Motility	50	67.3
Minimal pH for growth		
5.0	50	96.0
Terminal pH 4.5 to 4.7	50	96.0
Fermentation of		
α-Methyl-D-glucoside	50	100
Raffinose	50	100
Xylose	50	100

The authors state that the organism has been isolated from edible greens, legumes, corn plants, grasses, cucumbers, several types of squash, maturing grains, broccoli, and a variety of leaves and vegetables. Levels range from near 0 to 10^6 per gram of plant material. They found that it is frequently the most numerous, and sometimes the only, member of the lactic acid-producing bacteria isolated from plants.

S. faecium var. casseliflavus is closely related to S. faecium because of essential properties such as the dissimilation of malate, production of peroxidase

but not of catalase, and the fermentation of arabinose, melibiose, and salicin, but not of melezitose. The organism's status as a variant is justified because of the factors of pigmentation and ecology but, more importantly, because of the high pH at which growth is initiated and terminated, as compared with the limits for the enterococci. The majority of the cultures are motile, whereas true enterococci are not (Deibel, 1964). These pigmented bacteria ferment raffinose (a sugar fermented also by S. bovis), as well as α-methyl-D-glucoside and xylose. Neither S. faecalis nor S. faecium can ferment these sugars. In addition, S. faecium tolerates a high temperature of incubation; the variant does not. The variant grows rapidly at 10°C whereas four or more days are required by S. faecalis and S. faecium. There are seasonal differences as well. The pigmented bacteria thrive during the spring and early summer months, and again in midfall, whereas the enterococci are sparse in the natural environment until midsummer, after which both incidence and numbers increase.

In the Streptococcaceae yellow, cell-bound pigments are known to occur only in group D. Taylor et al. (1971) found that pigments extracted from three strains of yellow enterococci showed the spectral and solvent partition characteristics of carotenoids. The properties of the enterococcal carotenoids are similar to those found in Flavobacterium, Xanthomonas, Micrococcus, Sarcina, Corynebacterium and Cellulomonas species. Taylor's group observed that yellow pigmentation in the group D streptococci with which they worked appeared to vary from organism to organism independent of other characteristics, thus rendering pigmentation of questionable value for taxonomic purposes.

Gas chromatography has been used (Amstein and Hartman, 1973) to compare the relative fatty acid composition of different strains of enterococci, including S. faecalis, S. faecium, S. faecium subsp. durans, and three strains of S.

faecium subsp. casseliflavus. The authors concluded that, based on differences between the fatty acid composition of strains of S. faecium subsp. casselifalvus and that of the other strains, S. faecium subsp. casseliflavus deserved varietal (now subspecies), if not species, status.

Roop and coworkers (1974) using deoxyribonucleic acid (DNA) - DNA hybridization, noted that a strain of S. faecium subsp. casseliflavus exhibited little homology with reference strains of S. faecium, S. faecalis, and S. lactis. Deoxyribonucleic acid (DNA) homology was also used by Vaughan et al. (1979) to assess genetic relatedness among yellow-pigmented strains of group D streptococci and to examine possible genetic relatedness of these strains to S. faecium and S. faecalis. On the basis of tests, the yellow-pigmented strains were divided into two groups, I and II. Unlike group I, the DNAs of group II strains exhibited high thermal dissociation temperatures (Tm's) and had a high degree of homology with the DNA of the type strain (ATCC 25788) of S. faecium subsp. casseliflavus. The group II organisms also grew in the presence of 6.5% NaCl, and were not able to ferment sorbitol or decarboxylate tyrosine. The authors proposed that the group II strains should constitute a separate and distinct species with the name Streptococcus casseliflavus (Mundt and Graham) comb. nov.

Isolation Methods

Volterra et al. (1986) reported that at least 44 agarized media and 33 broths have been developed for the selective isolation of fecal streptococci.

In 1989 the International Organization for Standardization (ISO) proposed the following methods for the detection and enumeration of enterococci. The media suggested for use with membrane filtration were KF-streptococcus agar (Kenner) and m-enterococcus agar (Slanetz and Bartley). Bile-aesculin-azide

agar (BEA) was proposed as a confirmation medium. Additionally a catalase test should be performed on suspect colonies. The characteristics of the Enterococcus and Streptococcus species are provided in the Table below.

Table 2. Enterococcus and Streptococcus species, their occurrence in feces and characteristics.

	Occurrence in feces	D	Growth on KF	Growth on BEA 44°C
Enterococcus				
E. avium	+	+		+
E. casseliflavus	+	+		+
E. durans	+	+	+	+
E. faecalis	+	+	+	+
E. faecium	+	+	+	+
E. gallinarum	+			+
E. hirae	+	+		
E. malodoratus		+	+	-
E. mundtii	+	+	+	
E. pseudoavium				
E. raffinosus		+		
E. solitarius		+		
Streptococcus				
S. alactolyticus		+		
S. bovis		+-		
S. canis		-		
S. cecorum		-	+	
S. equinus		+	+	
S. cricetus				
S. ferus				
S. intermedius		-		
S. mutans				
S. rattus				
S. saccharolyticus		-		
S. salivarius		-		
S. sobrinus				

Standard Methods for the Examination of Water and Wastewater, also published in 1989, suggests mE agar (Levin et al., 1975) and m Enterococcus agar (Slanetz and Bartley, 1957) for isolation of enterococci and fecal streptococci, respectively.

Pagel and Hardy (1979) compared five media for their recovery of fecal streptococci on membrane filters. Of the five (KF agar, Slanetz and Bartley (SB) medium, mE, m Enterococcus, and Pfizer selective enterococcus agars), the best overall results were obtained on m Enterococcus agar.

Fecal Streptococcal Survival

a) Viability in Water

The effect of storage of water samples on the viability of fecal streptococci at 8°C and at room temperature (26°C) was studied by Bartley and Slanetz (1960). They found that little change in numbers of fecal streptococci and coliforms occurred when the samples were stored at 8°C for periods up to two days. After a one-day storage period there was a definite decrease in numbers of these organisms in samples stored at 26°C.

The authors also suspended known numbers of bacteria from recently isolated strains of S. faecalis, S. faecalis var. liquifaciens, S. faecalis biotype 1, S. faecium and S. bovis in dialyzing bags and immersed the bags in fresh water in a river and in salt water in a bay at 20°C temperatures. The numbers of viable cells decreased from 25 to 75 percent during the first 24- hour period. This was followed by a less pronounced die-off for three to four days with only small numbers or none remaining viable after seven days. The most viable of the strains of fecal streptococci tested were S. faecalis biotype 1 and S. faecium.

In polluted water the coliform-Streptococcus ratio generally is significantly greater than unity; however, the streptococci occasionally may outnumber the coliforms. After initial contamination, both groups may exhibit a slight increase in numbers (presumably as a function of the organic material available in the water and the temperature) followed by a pronounced decrease (Bartley

and Slanetz, 1960). A study of the coliform - Streptococcus ratio in various water sources by Burman (1961) indicated a more rapid decrease in coliforms, and a marked tendency was noted for the ratio to approach unity. The group D streptococci were not detected in waters known to be free from fecal contamination.

The rapid disappearance (as determined by agar counts) of fecal bacteria released into natural aquatic environments has been demonstrated on a number of occassions over the last 100 years (Aubert et al., 1981, De Giaxa, 1889, Mancini, 1978). The effect of environmental factors, such as salinity, temperature, light, and the presence of predators, on the rate of disappearance has also been described previously (Aubert et al., 1981; Carlucci and Pramer, 1959; Mitchell and Chemberlin, 1975; Mitchell and Morris, 1969; Orlob, 1956).

Recently the validity of the classical enumeration techniques in which selective media are used have been questioned. For example, Colwell and co-workers (Colwell et al., 1985; Grimes and Colwell, 1986; Palmer et al., 1984; Roszak et al., 1984; Xu et al., 1982) found that when enteric bacteria are introduced into seawater, they maintain cellular integrity for several days to several weeks but rapidly lose their ability to be cultivated by classical methods. When the nonculturable cells are examined for metabolic activity, they are viable, and in some instances they have been found to have retained their virulence (Colwell et al. 1985; Grimes and Colwell, 1986). These findings suggest that the rate of loss of culturability on the usual agar media strongly underestimates the risk of survival of these bacteria.

Garcia-Lara et al. (1991) developed a procedure for determining the mortality rate for allochthonous bacteria released in aquatic environments without interference due to the loss of culturability in specific culture media. Their method follows the disappearance of radioactivity from the trichloroacetic

acid-insoluble fraction in water samples to which [^{3}H] thymidine-prelabeled allochthonous bacteria have been added. Using the technique they measured the rate of disappearance of E. coli, Salmonella typhimurium and S. faecium at 11°C. Their results showed that the overestimation of mortality rates by agar plating methods was less pronounced for S. faecium than for the other two organisms.

b) Survival in Soil

Persistence of enteric organisms in soil following contamination is of concern in the evaluation of indicator organisms. Mallmann and Litsky (1951) tested the survival of S. faecalis, Salmonella typhi, and E. coli in a number of soils which were contained in metal cylinders. The temperature range during the study was 25 to 28°C. E. coli was still recovered after 11 weeks, whereas S. faecalis persisted for approximately 40 days and S. typhi survived for a maximum of 18 days.

Two outdoor test areas were periodically inoculated with test strains of E. coli and S. faecalis in a survival study conducted by Van Donsel et al. (1967). A significant seasonal variation in survival was observed. During the summer, fecal coliforms outlived fecal streptococci (fecal streptococci survived 2.7 days, whereas fecal coliforms remained viable for 3.3 days). In the autumn, survival time was approximately 13 days for both species. During the winter and spring, fecal streptococci persisted for as long as 20 days, much longer than fecal coliforms.

c) Survival Studies in Frozen Foods

Bacterial surveys of commercially frozen foods showed that fecal streptococci could be isolated from a wide variety of vegetable samples. Citrus concentrates

had fewer enterococci than the vegetable samples examined (Larkin et al. 1955a). Hahn and Appleman (1952a, b) inoculated stock cultures of E. coli, Salmonella typhosa, and Shigella paradysenteriae into pasteurized orange concentrate. When the concentrate was frozen the bacterial numbers were so reduced at the end of 24 hours that a plate count could not be obtained. Streptococcus faecalis added from fresh fecal material or from stock cultures outlived all other enteric organisms.

Streptococcus faecalis and S. liquefaciens inoculated into fresh green beans survived storage at temperatures of 0, -5, and -20°F for 400 days. The organisms were found to neither increase nor decrease in numbers significantly during a storage period of 217 days at 0°F. On the other hand, E. coli showed at least a fivefold decrease in numbers during storage at the same temperature for the same time (Larkin et al., 1955b).

Streptococci in the Human Host

In humans Streptococcus is considered to be important as a lactic acid producing microorganism. The streptococci constitute a major group of the lactic acid bacteria along with bifidobacteria in the ileum and the large intestine (Kawai et al., 1980). Streptococci and bifidobacteria are considered to be more important than lactobacilli as a part of the lactic acid producing intestinal microflora. There are some reports on the classification, distribution, and colonization of streptococci in human feces but, in general, the exact speciation of the enterococci from this source has been largely neglected.

Previous studies have indicated that S. faecalis subsp. liquefaciens constitutes about 25 percent of the streptococcal population in human feces (Geldreich and Kenner, 1969; Cooper and Ramadan, 1955). According to Cooper

and Ramadan (1955) typical S. faecalis comprised 40 percent of human-derived streptococci but could not be isolated from cow and sheep feces. S. faecalis (Bartley and Slanetz, 1960), S. durans (Cooper and Ramadan, 1955), S. mitis and S. salivarius (Kenner et al., 1960) have all been reported to be unique to human feces. The latter two are buccal streptococci that populate feces in low numbers when they enter the digestive tract. S. bovis and S. equinus are rarely found in human stools.

Lamka (1979) obtained streptococcal isolates from raw sewage, fresh fecal specimens of domestic animals as well as soil and vegetation and quantitatively determined the biotypes present in each. S. salivarius was found to comprise 25% of the organisms isolated from raw sewage, but was absent in animal feces, soil and vegetation samples. Distinct differences were noted between a protected forest area and an area receiving runoff affected by man's activity. Thirty percent of the biotypes were S. salivarius, and thus of human fecal contamination, in the runoff of the nonprotected area, whereas no S. salivarius was found in that of the limited access forest.

More recently, Kawai and coworkers (1980) investigated the distribution of human fecal streptococci at the species level. The percentage isolation of each streptococcal species from the feces of children and adults is shown below.

Streptococcal strain	Number of strains isolated from Adult (n=26)	Child (n=25)
S. faecalis	97	75
S. faecium	196	118
S. durans	48	17
S. bovis	86	244
S. equinus	24	19
S. avium	0	204
S. salivarius	191	9
S. mitis	43	7
S. MG-intermedius	37	4
S. sanguis II	1	0
Unknown	18	4
Total	741	701

The clear differences in the streptococcal distribution between the two age groups prompted the authors to divide the isolates into three categories: child type (S. bovis and S. avium, adult type (S. salivarius, S. mitis, and S. MG - intermedius), and ubiquitous type (S. faecalis and S. faecium).

In the same study Kawai et al. administered eight species of streptococci, four species of lactobacilli and four species of bifidobacteria to a germ-free rat and sampled human contents and the walls of digestive tracts of the animals one week later. The streptococcal population level was high in all the human contents (10^5 to 10^9/g feces), and walls of the jejunum (10^4/g tissue), ileum (10^6 to 10^8/g tissue), and cecum (10^8/g tissue) indicating that streptococci commonly isolated from humans can become established in rats among lactic acid bacteria particularly in the lower digestive tract of the rat.

S. faecium subsp. casseliflavus has not been cited in the literature as being part of the streptococcal flora of human feces. This is consistent with

our finding that S. casseliflavus is commonly found in storm sewer water but can only be isolated in very low levels from sanitary sewage (Seyfried, 1990). To date we have not been able to recover this organism from human fecal samples.

Distinguishing Human from Animal Pollution

Sanitarians have continually been seeking some criterion by which sewage pollution from human origin could be distinguished from the surface wash of streets and agricultural land. As early as 1910 Winslow and Palmer attempted to determine whether a general distinction could be made between human and fecal streptococci. They found the characteristic differences between S. bovis, S. equinus and S. faecalis, but only since the fifties has it been possible to differentiate S. faecium from S. faecalis (Skadhauge, 1950).

Studies by Bartley and Slanetz (1960) on the types of streptococci present in human and animal feces indicated that it might be possible, in some instances, to distinguish between human and animal fecal contamination. They suggested that, while typical S. faecalis may not always be the predominant type of streptococcus in human feces, it is not found in the feces of most domestic animals. Therefore its presence in water would indicate contamination of human origin. They also stated that the raffinose fermenting, tellurite resistant fecal streptococci appear to be of animal origin, particularly cows and sheep. Furthermore, the presence of S. bovis in a water sample would suggest recent bovine or ovine pollution.

Streptococci in Domestic Animals

Regardless of their diet, the feeding habits of all animals provides ample opportunity for the implantation of enterococci in the alimentary canal.

Nonetheless, extremely low numbers or even total absence in some animals suggests a selective action of the digestive tract upon these bacteria (Mieth, 1962).

Of all the domestic animals, the pig is closest to humans in terms of digestive tract similarities (L.W. Macpherson, D.V.M., personal communication). Mundt (1963) observed that domestic swine, similar to wild boar, harboured a higher incidence of S. faecium over S. faecalis. Fewins et al. (1957) isolated S. faecium from a pig on a grass diet and Barnes et al. (1956) showed that S. faecium was normally present in the gut of the pig and could be isolated from spoiled canned hams.

Medrek and Barnes (1962) studied the serological and physiological properties of 153 strains of S. bovis isolated from cattle and sheep. They found that generally more than one and up to four types occurred in a given animal. In addition, several common types occurred in cattle and sheep suggesting that there was no host specificity. When fecal samples from four cows were examined over an 18 month period there was no evidence that a change from winter to summer diet influenced the numbers of S. bovis although there was a change in type with passage of time. A similar change in type was observed by Emslie-Smith (1961) when the E. coli content of human feces from one adult was examined at frequent intervals over several months.

In another study Medrek and Barnes (1962a) determined the numbers and types of Lancefield group D streptococci in the colons of 17 cattle and 9 sheep. The mean total streptococcal counts were 8×10^4 per gram in cattle and 2×10^6 per gram in sheep. Every sample contained S. bovis and it was the predominant species in 15 of the cattle and 6 of the sheep. Streptococcus faecalis, S. faecium and S. durans were rarely isolated from cattle but they formed a significant proportion of the population in sheep. Fifty-one of the

60 S. faecium, S. durans and related strains fermented raffinose.

Bartley and Slanetz (1960) noted the rare occurrence of S. faecalis in domestic animals, with the exception of chickens. In poultry S. faecalis was found in larger numbers, although S. faecium and "S. inulinaceus" were also frequently encountered.

Streptococci in Wild Animals

Variation in the group D streptococcal flora of animals has been associated with geographical location, diet, age, and species of the host. Seasonal effects have also been observed in some instances. Differences in the predominant flora of the same host examined at different time intervals have been reported as well.

The incidence of enterococci in wild mammals, reptiles and birds in a native state was surveyed by Mundt (1963). It was found that 71% of the 216 various mammalian species studies harbored enterococci. A higher incidence of S. faecium over S. faecalis was noted in wild boars but, in general, S. faecalis was isolated most frequently from the feces of other wild mammals. Enterococci were isolated only sporadically from rodents and then only in low numbers so that the possibility of these animals being natural hosts is doubtful (Ostrolenk and Hunter, 1946).

Mundt (1963) found that a large number (85%) of reptiles contained enterococci and the incidence of S. faecalis was superior to S. faecium. Although an extensive survey of avian species was not attempted, 7 of 22 specimens tested yielded enterococci. Needham et al. (1979) collected fresh droppings from six species of captive birds of prey, i.e. kestrel, buzzard and four types of owl. They found that 44 to 75% of the samples yielded enterococci.

Streptococci in Plants, Soil and Insects

The question of the incidence of fecal streptococci on plants should be addressed for two reasons. One important consideration is the fact that streptococci on plants may be introduced into soil or water. Recovery in water of large populations of group D streptococci from plants could yield inconclusive results if these organisms were used as fecal pollution indicators. Also, the recovery of enterococci from processed vegetables cannot be considered indicative of fecal contamination if the organisms are natural residents of plants.

Sherman in 1937 noted the occurrence of enterococci on plants and suggested that some growth take place. More recently Mundt and coworkers (1958, 1962) confirmed the common occurrence of enterococci, particularly on domestic plants. Since the bacteria could establish a cycle in plants with transmission in the plant seed (1962), an epiphytic relationship was proposed. Mundt (1963) also concluded that the enterococci occurring on plants arise commonly from wild animals, and that they do not represent plant-specific species or variants of the enterococci. Further work by Mundt et al. (1967) showed that streptococci producing a pale, lemon-yellow pigment on clear media were most numerous on vegetables during the spring and early summer months. He suggested that enterococci may be regarded as temporary residents on plants; they are capable of limited reproduction and they are disseminated among plants by the action of insects and wind, and spread to the ground by these agencies, gravity and rain (Mundt, 1961).

Gildreich and co-workers (1964) also reported the recovery of enterococci from plants. Of the 646 enterococcal isolates tested, 37.7 percent were able to hydrolyze starch. This characteristic was uncommon in human and other isolates. Other researchers were able to isolate enterococci from silage

(Mieth, 1961) and from the seeds of corn and peas (Fitzgerald, 1947).

The fact that fecal streptococci are found less frequently on plants growing in the wilderness would indicate that plant populations originate from a fecal source (Clausen et al., 1977). However, the unique biotypes characteristic of plants suggest that residual populations on certain plants provide a reservoir that may be spread to other vegetation, possibly by insects.

It is now generally agreed that enterococci are not native to soil; their presence in soil samples represents contamination from either animal or plant sources. Medreck and Litsky (1960), for example, found that only 8 of 369 undistrubed soil samples contained enterococci. Mundt (1958) observed a significantly higher incidence in cultivated soils and adjacent areas and he associated this with enterococcal growth on the plant host.

Streptococcus faecalis var. liquefaciens has been found in connection with vegetation, farm soil (Geldreich, 1976), insects (Martin and Mundt, 1972) and fish, in addition to human and animal feces. The organism grew in comparable numbers and produced the same type of colony as S. faecalis on the isolation media assessed by Pagel and Hardy (1980).

Fecal streptococci have been isolated from a wide variety of insects including Hemiptera (true bugs), Orthoptera (grasshoppers), Lepidoptera (moths and butterflies), Coleoptera (beetles), Hymenoptera (bees, wasps, ants), Diptera (flies), and Homoptera (leaf hoppers) (Geldreich et al., 1964; Martin and Mundt, 1972; Steinhaus, 1941; West, 1951). Geldreich et al. (1964) found that the fecal streptococcus population isolated from five orders of insects was composed of 52 percent enterococci and 48 percent S. faecalis var. liquefaciens. Streptococci from eight insect orders were speciated by Martin and Mundt (1972). They found that S. faecalis could be recovered from 32 percent, S. faecium

from 22 percent and S. faecium subsp. casseliflavus from 44 percent of the insects examined.

Eaves and Mundt (1960) studied the identity and distribution of enterococci associated with insects. They found no pattern of species distribution and the streptococci occurred randomly. They therefore concluded that the association was circumstantial. The enterococci appear to be transient residents in the insect digestive tract and their occurrence on the insect exterior is probably due to mechanical transfer.

It is clear from the studies conducted thus far that more work is necessary before the relationship between streptococci and plants, soil, and insects is fully understood.

Streptococci as Indicators of Fecal Contamination

Barnes et al. (1956) stated that S. faecium has a wide distribution among animals and may also be found in man whereas S. faecalis seems to be much more restricted to the gut of man. For this reason they proposed the use of S. faecalis instead of the coli-aerogenes bacteria as an index of faecal contamination. Other authors (Frenchem, 1975; Geldreich, 1976) have suggested that water samples be analyzed for both fecal coliforms and fecal streptococci to determine whether fecal contamination is from human or other sources.

Although fecal streptococci have been proposed as an indicator of drinking water quality, Ptak and Ginsburg (1977) reported that the data presented at the ASTM International Symposium on Bacterial Indicators of Potential Health Hazards Associated with Water in June, 1976 show that coliforms are better suited as indicators. The fecal streptococci tend to die off rapidly in water of good quality and are not as numerous as coliforms. The authors suggest that enterococci

might be better indicators of pollution in wells (animal contamination), swimming pools, and sewage treatment plants (high in nutrients, low in oxygen).

In another study, St.-Louis and Legendre (1982) used the bacteriological data gathered over a nine-year period from three Quebec lakes to develop a microbial water quality index. The three variables included in the index were total coliforms, fecal coliforms, and streptococci. The index was then used to examine the time variations of water quality on the various beaches, graphically for weekly fluctuations and by linear regression for the long-term variability.

The most recent edition of Standard Methods (APHA 1989) strongly supports the use of the enterococcus group as a bacterial indicator for determining the extent of fecal contamination of recreational surface waters. The book cites studies at marine and fresh water bathing beaches indicating that swimming-related gastroenteritis is directly related to the quality of the bathing water. The data showed that enterococci are the most efficient bacterial indicator of water quality (Cabelli, 1983; Dufour, 1984). The U.S. Environmental Protection Agency has proposed water quality guidelines for recreational waters based on enterococcal density (U.S. EPA, 1986). The guideline is 33 enterococci/100 mL for recreational fresh waters and 35/100 mL for marine waters. The guidelines are based on the geometric mean of at least five samples taken over a thirty-day period during the swimming season.

Other Water Quality Indicators

Coliphage

Gerba et al. (1975) have suggested that over 100 different types of viruses can occur in domestic sewage. Although a number of outbreaks of infectious hepatitis and possibly poliomyelitis have been traced to fecal contamination of

waters (Craun and McCabe, 1973; Bryan et al., 1974; Goldfield, 1976; Gerba and Goyal, 1978) transmission of enteroviruses by the water route is difficult to establish.

The sanitary quality of water is currently judged on the basis of indicator bacteria; however, these bacteria may not accurately reflect the virological quality of water. For example, enteric viruses are known to be more resistant to commonly used wastewater and water treatment methods (including disinfection) than are enteric bacteria (Berg et al., 1978). The inactivation rate in these processes is higher for bacteria than for viruses.

Marzouk et al. (1980) monitored the aquatic resources of Israel to obtain background information on the occurrence of enteroviruses and to determine how adequately bacteriological indicators reflected their presence. They examined a total of 155 samples of groundwater as well as surface, potable, and swimming pool water, of which 45 (29%) yielded virus. Echovirus type 7 was isolated most frequently, followed by poliovirus 1, echovirus 6 and coxsachieviruses B2, B5 and B6. Six of seven swimming pool samples yielded virus even though three of the six pools had a chlorine residual. All isolations occurred when the population of fecal coliforms in the pools was less than 100 per 100 mL. Viruses were also isolated from water samples with no detectable fecal or total coliform bacteria. The authors found that no significant statistical correlation could be determined between the occurrence of bacterial indicators and the presence of viruses.

Bacteriophages, particularly coliphages, have been examined as indicators of virus pollution. For example, the male specific RNA coliphages such as F-2 (Scarpino, 1975; Loeb and Zinder, 1961; Kott et al., 1974) have been proposed as water quality indicators because their size, shape, and chemical composition

are similar to those of the enteroviruses (Scarpino, 1975). In addition, their resistance to chlorine is similar to the enteroviruses (Scarpino, 1975; Loeb and Zinder, 1961; Kott et al., 1974; Dhillon and Shillon, 1974; Olivieri et al., 1975) and during sewage treatment their levels parallel enterovirus levels (Cabelli, 1977).

The F specific RNA bacteriophages are useful models for the study of the fate of animal viruses in water treatment processes; pure cultures of MS2 and f_2 have frequently been used in model experiments. These male specific bacteriophages adsorb to F-pili and thus can only infect male host strains. A method has been developed for the selective enumeration of these phages using a male Salmonella strain, constructed by the introduction of the plasmid F '42 lac :: Tn 5 into Salmonella typhimurium phage type 3. A number of authors (Snead et al., 1980; Berg, 1983; Shah and McCamish, 1972; Cramer et al., 1976; Drulak et al. 1979; Bausum et al., 1982) have reported that these viruses that adsorb to F pili as the first stage in infection are highly resistant to disinfectants.

The f_2 bacteriophage was used by Keswick, et al. (1985) to demonstrate the inactivation of viruses in drinking water by chlorine. The studies showed that, with the exception of bacteriophage f_2, the poliovirus, rotavirus and Norwalk viruses were completely inactivated by similar doses of chlorine. The f_2 phage appeared to be more resistant than the viruses to specific chlorine doses. Norwalk virus and f_2 bacteriophage were found to survive for 30 min. in the presence of residual free chlorine. The references cited by these authors indicated that Norwalk virus and hepatitis virus are somewhat more chlorine resistant than are other enteroviruses.

The somatic coliphages comprise all tailed and cubic DNA bacteriophages

capable of infecting an E. coli C host strain by adsorption to receptors in the cell envelope.

The first researcher to note the potential of bacteriophages as indicators of fecal pollution was Guelin in 1948. Since then, a number of papers have recognized the potential of bacteriophage/coliphage to serve as indicators of bacterial water quality (Wentsel et al., 1982: Kennedy et al., 1985) and viral water quality (Kott et al., 1978; Grabow et al., 1984).

A study by Scott et al., (1979) showed that Escherichia coli cells are nonviable after a 5-minute contact with 6 mg of chlorine per litre while coliphages survive an exposure to 25 mg of chlorine per litre for 80 min. Thus, coliphages appear to be more resistant to inactivation by chlorination than are coliforms.

It has been suggested by El-Abagy et al. (1988) that the coliphage test has numerous advantages over traditional bacteriological and virological tests because the procedure is economical, simple to perform and provides results within 6 hours.

Pseudomonas aeruginosa

The incidence of Pseudomonas aeruginosa in human fecal specimens was assessed by Sutter et al. in 1967. They found that approximately 12% of healthy adults or employees in medical or dental schools discharged the organism in numbers up to 10^5 g $^{-1}$ of feces. Pseudomonas aeruginosa appeared persistently in some individuals but only transiently in others. A number of researchers (Bonde, 1963; Drake, 1966; Hoadley, 1967; Cabelli et al., 1976; and Jawad, 1976) have observed a higher incidence of the organism in heavily polluted waters than in waters with comparatively low levels of pollution. All have suggested that it might be used as an indicator of fecal pollution.

Wheater et al. (1980) determined the incidence of P. aeruginosa in sewage from domestic and hospital sources and in waters having different levels of sewage pollution. They found P. aeruginosa could be isolated consistently from domestic and hospital sewage. The numbers were usually less than 3.0×10^4 per 100 mL in domestic sewage whereas counts above 5.0×10^5 per 100 mL were frequently obtained in hospital sewage. Correspondingly high counts of E. coli were not found in hospital sewage and the ratio of E. coli to P. aeruginosa was usually less than 25 compared to ratios of over 500 in ordinary domestic sewage. Levels of domestic pollution were also reflected in the numbers of P. aeruginosa in fresh-water stream samples. Variations in the counts of the organism over the length of the stream closely paralleled those of E. coli. However, P. aeruginosa was frequently not isolated from samples taken from the stream in lightly populated, agricultural areas even when the counts of E. coli were high. This was consistent with results from examinations of fecal specimens which confirmed the presence of P. aeruginosa in humans but indicated that in animals the organism was not present in significant numbers. P. aeruginosa was recovered from three out of a total of 20 human fecal specimens with counts ranging from 5.7×10^2 to 5.0×10^3 per gram. The organism was found in one of the 24 pigs examined (count = 1.5×10^2) but was not recovered from any of the 35 cattle or 30 sheep or from a variety of other domestic animals and rodents.

Bifidobacterium sp.

Taxonomy and Nomenclature

Bifidobacteria are gram-positive, nonsporeforming anaerobic rods which are common to the digestive systems of many higher animals. They have a bifurcating pleomorphic cellular morphology and also degrade glucose anaerobically to acetic and lactic acids through a peculiar fructose-6-phosphate shunt pathway (de Vries *et al.*, 1967; Scardovi and Trovatelli, 1965).

Despite the fact that bifidobacteria were first described in 1899 (Tissier, 1899), they have been the least studied of all the potential indicators of fecal pollution. Poupard and coworkers reviewed the biology of the genus in 1973 (Poupard *et al.*, 1973), and the most recent classification of the organism appears in the 1986 edition of Bergey's Manual of Determinative Bacteriology (Scardovi, 1986) in which 24 species are described.

Characterization of Bifidobacteria

The phenotypic traits which distinguish species of the genus *Bifidobacterium* include gross morphology, fermentation characteristics, guanine plus cytosine content of the DNA, the interpeptide bridge of the cell-wall peptidoglycan, and the transaldolase and 6-phosphogluconate dehydrogenase isozyme pattern (determined by starch gel electrophoresis).

Scardovi *et al.* (1979) described four new species of *Bifidobacterium* using the competition filter method to determine DNA-DNA hybridization.

Enzymic tests and amounts of acetic and lactic acids produced were used by Chevalier *et al.* (1990) to differentiate the bifidobacteria from the lactobacilli used in dairy products. Suspensions of the organisms in deionized water were added to the 19 dehydrated chromogenic enzyme substrates in API ZYM kits obtained from Analytab Products (Plainview, N.Y., U.S.A.). The results showed that two enzymes, α-galactosidase and glucosidase were present in almost all bifidobacteria tested. All the *Lactobacillus* strains studied were α-galactosidase negative. The most direct and reliable characteristic assigning an organism to the genus *Bifidobacterium* is based on the demonstration of F6PPk. Physiological studies of bifidobacteria have shown that glucose is degraded exclusively by the fructose-6-phosphate shunt in which fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22) cleaves fructose-6-phosphate which later gives rise to acetic and lactic acids in the theoretical ratio of 3:2.

Work has begun in our laboratory to establish if the API Rapid CH test system can be used to effectively determine fermentative reactions of different *Bifidobacterium* spp. The Rapid CH kit, containing 49 sugars, could prove to be a useful tool for species differentiation.

Isolation Methods

Although bifidobacteria were proposed as potential indicators of fecal contamination in 1958 (D.A.A. Mosel, Abstr. 7th Int. Congr. Microbiol., 1958, p. 440), little research has been done because of the lack of a suitable medium

for selectively enumerating these organisms and the difficulty with anaerobic methodology.

Gyllenberg and Niemela (1959) developed a medium for bifidobacterial enumeration but this medium has not been evaluated in the literature. A modification of this medium, used in studies by Evison and James (1973; 1974), has also not been sufficiently evaluated. The presence of triphenyltetrazolium chloride in the medium causes the colonies to become dark red in colour and easy to distinguish. However, work by Levin's group (Levin, 1977) showed that not all of the dark red colonies are bifidobacteria. They found that, depending on the sample source, the percentage of false positives ranged from 20 to 55 percent, most of which were identified as lactobacilli. As well, the use of arabinose as the sole carbohydrate limits the medium to B. adolescentis, B. asteroides, B. coryneforme, B. longum, B. pseudolongum, and B. suis. Three of the species known to be associated with human feces, i.e. B. bifidum, B. infantis, and B. breve, would not be recovered.

Other selective media were developed for the isolation of bifidobacteria (Finegold et al. 1971; Mitsuoka et al., 1965). Antibiotics such as neomycin, kanamycin, or nalidixic acid were used as the primary slective agents in most of these media. However, it was shown by Finegold et al. (1971) that any antibiotic system incorporated into conventional nonselective media was not fully selective and often failed to promote growth of bifidobacteria.

A simple synthetic medium consisting of lactose, ammonium acetate, cystine, biotin, and pantothenic acid was proposed by Petuely (1956) in 1956 for the isolation of Bifidobacterium from human fecal samples. Tanaka and Mutai (1980) improved Petuely's medium by addition of riboflavin, nucleic acid bases, pyruvic acid, and nalidixic acid. The medium was designed to meet the nutritional requirements of bifidobacteria and the authors showed that the concentration of 100 ug/mL nalidixic acid suppressed almost all non-Bifidobacterium strains.

In 1981, Resnick and Levin (1981) described a membrane filter technique developed for the enumeration of bifidobacteria in natural aquatic environments. The medium used (YN-6) contained nalidixic acid, neomycin sulfate, and bromcresol green as selective and differential agents. The authors compared the YN-6 medium with the medium of Gyllenberg and Niemela (1959) and showed that the YN-6 method produced superior selective recovery of bifidobacteria. However, it was found that the method did not adequately repress gram-positive facultative cocci.

A few years later, Mara and Oragui (1983) published the formulation for YN-17 medium, which was a modification of Resnick and Levin's YN-6. The medium contained two additional antibiotics, kanamycin sulphate and polymyxin B. Kanamycin sulphate was shown to substantially reduce the proportion of contaminating faecal streptococci; however, it should be noted that complete inhibiton was not achieved.

Munoa and Pares (1988) reconfirmed in their paper that YN-6 medium is inhibitory to some species of Bifidobacterium and is not sufficiently selective to provide a real estimate of the number of viable cells of bifidobacteria. While YN-17 is more selective than YN-6, it inhibits a part of the viable bifidobacterial population (Munoa et al., 1985). When using these two media,

further confirmation of the isolated colonies is necessary to differentiate Bifidobacterium spp. from other organisms (Carrillo et al., 1985; Mara and Oragui, 1983; Munoa et al., 1985). A new selective and differential medium, Bifidobacterium iodoacetate medium 25 (BIM-25) was developed by Munoa and Pares (1988). It is composed of reinforced clostridial agar, nalidixic acid, polymyxin B sulfate, kanamycin sulfate, iodoacetic acid, and 2,3,5-triphenyltetrazolium chloride. On this medium all red colonies with a diameter of less than 2 mm were formed by gram-positive cocci that grew well under aerobic conditions. White colonies with a diameter exceeding 2 mm were always Bifidobacterium spp. Pink colonies were produced by cocci, bifidobacteria, and other rods. To improve the recovery of injured bifidobacteria, the authors incorporated the resuscitative technique of solid medium repair (Andrew and Russell, 1984) into the procedure. The filters were first incubated anaerobically on reinforced clostridial agar for 5 hours and then transferred to BIM-25. This step produced injury repair in all strains tested.

Beerens (1990) described a modified Columbia agar medium containing 5 or 10 mL/L propionic acid and adjusted to pH 5.0. According to the author, the combination of low pH and propionic acid makes the medium both elective and selective. The following year Beerens (1991) introduced the use of the membrane filtration technique and the enrichment method to recover bifidobacteria from a variety of animal species. Filters were incubated on plain Columbia agar for 6 hours at 37°C under anaerobic conditions before transfer to the modified Columbia agar medium.

The literature suggests that, as yet, there is no ideal medium for isolation of Bifidobacterium spp. from environmental samples. However, an appropriate resuscitation procedure will clearly enhance the recovery of bifidobacteria.

Survival Studies

A number of studies have suggested that E. coli may be able to survive and regrow for extended periods in tropical habitats (Bonde, 1977; Evison and James, 1973; Carillo et al., 1985). However, E. coli does not survive well in freshwater in temperate environments (McFeters et al., 1974; McFeters and Stuart, 1972; Sjogren and Gibson, 1981). In contrast, in tropical environments B. adolescentis densities decreased significantly over time in diffusion chambers (Carrillo et al., 1985). The B. adolescentis density decline was more than 50% in 48h. Bifidobacteria in the laboratory (held in filter-sterilized freshwater at 4, 12, and 20°C) declined more than 80% in cell density in only 24h (Resnick and Levin, 1981). Thus the in situ conditions of the tropical rain forest river appear to be much more conducive to prolonging bifidobacteria survival. Site 1, which possessed higher nutrient levels (i.e., total phosphorus, phosphates, and nitrates plus nitrites), had lower densities of E. coli but higher rates of survival of B. adolescentis. A possible explanation might be that, because there was a slight nutrient advantage for B. adolescentis, microbial antagonism was a major factor. It has been reported that bifidobacteria and other anaerobes can inhibit E. coli (Miller and Finegold, 1967; Poupard et al., 1973).

A 1960 report by Gyllenberg and coworkers (Gyllenberg et al., 1960) stated that there was no difference between the survival of bifidobacteria and coliforms at room temperature. Our studies, on the other hand, showed that E. coli

survived for 48h in sterile Lake Ontario water at room temperature whereas Bifidobacterium breve was nonviable after 24h (Seyfried et al., 1990).

Bifidobacteria in the Human Host

The human fetus exists in a sterile environment until birth. After birth a rapid colonization of the intestinal tract occurs. With breast-fed infants the fecal flora consists mostly of bifidobacteria and about 1% of E. coli, enterococci, and lactobacilli (Mitsuoka and Kaneuchi, 1977). The fecal flora of bottle-fed infants, on the other hand, resembles that of adults. Bifidobacteria constitute the major flora, but aerobic bacteria such as E. coli and streptococci and anaerobic bacteria such as bacteroides, eubacteria and peptococcaceae are present in much higher numbers than in breast-fed infants (Mitsuoka, 1982).

The microflora of children resembles that of adults; the number of bacteroidaceae, eubacteria, and peptococcaceae may be greater than that of bifidobacteria, and streptococcal and E. coli numbers decrease to less than 10^8 per gram of feces (Mitsuoka and Hayakawa, 1972).

Bifidobacteria levels often decrease in older people whereas lactobacilli and Clostridium perfringens numbers tend to increase (Mitsuoka, 1982). Mitsuoka et al. (1974) found that there was a higher incidence of B. adolescentis and a lower incidence of B. longum in elderly persons. Benno et al. (1989) also showed that the number and incidence of B. adolescentis in elderly persons with a median age of 84 years in the Yuzurichara area of Japan was significantly higher than that in the elderly persons with a median age of 68 years in the Tokyo area. The diet of the elderly in Yuzurihara is characterized by a high intake of dietary fiber and this may have been responsible for the difference.

The constancy or stability of the intestinal flora has frequently been discussed (Bornside, 1978). Nonetheless, Drasar and Hill (1974) have suggested that the flora of the intestine can be altered by endogenous and exogenous factors such as: animal species, sex, age and habits. Physiological functions such as peristalsis, secretion of digestive enzymes, bile, mucus etc., adrenal function, immune mechanisms of the host, exogenous microorganisms, climate, diet, drugs, and emotional stress may also play a role. Acidity of gastric juices, peristalsis disorder, cancer or surgical operations on the stomach or small intestine, kidney or liver disease, pernicious anaemia, and blind loop syndrome have all been observed to be associated with disturbances of the intestinal flora.

There have been numerous articles concerning the beneficial effects of Bifidobacterium on its host (Nakaya et al., 1982; Tamura et al., 1984) but few studies on the interaction between enteric pathogens and bifidobacteria have been conducted. Nakaya (1984) was the first to demonstrate the protective activity of Bifidobacterium and its high molecular weight products for epithelial cells from invasion by Shigella. Further studies are necessary to explain the mechanisms of these protective activities and the mode of modification of epithelial cells by the active factors of B. infantis.

The characteristics of the bifidobacteria found in humans and other animals are as follows:

Table 3. Characteristics differentiating the species of the genus Bifidobacterium

Species	Electrophoretic pattern of enzymes F6PPK[a]	Immunological specificity group[b]	Found in[c]
1. B. bifidum	15	B	Feces of human infant and adult; human vagina; feces of suckling calf
2. B. longum	15	B	Feces of human adult and infant; (human clinical)
3. B. infantis	15	B	Feces of human infant; (human vagina)
4. B. breve	15	B	Feces of human infant and adult; (human vagina and clinical)
5. B. adolescentis	15	B	Feces of human adult; sewage; (rumen of cattle; feces of monkey and dog)
6. B. angulatum	15	A	Feces of human adult; sewage
7. B. catenulatum	15	A	Feces of human adult and infant; (sewage)
8. B. pseudocatenulatum		A	Feces of human infant and suckling calf; sewage
9. B. dentium	15	A	Human dental caries and clinical (feces of human adult and infant; human oral cavity and vagina)
10. B. globosum	10	E	Feces of pig, suckling calf, rat, rabbit and lamb; rumen of cattle; (sewage)
11. B. pseudolongum	10	E	Feces of chicken, cattle, rat and mice
12. B. cuniculi		C	Feces of rabbit
13. B. choerinum			Feces of piglet; (sewage)
14. B. animalis	10		Feces of rat, chicken, rabbit, calf; sewage
15. B. thermophilum	10	D	Feces of pig, piglet, chicken, calf, rumen of cattle; sewage
16. B. boum		D	Rumen of cattle; feces of piglet

Characteristics differentiating the species of the genus Bifidobacterium (cont'd.)

Species	Electrophoretic pattern of enzymes F6PPK[a]	Immunological specificity group[b]	Found in[c]
17. B. magnum	10	C'	Feces of rabbit
18. B. pullorum	10		Feces of chicken
19. B. suis	10	G	Feces of piglet
20. B. minimum	10	F	Sewage
21. B. subtile	10-15	F	Sewage
22. B. coryneforme	16		Intestine of Apis mellifera L. subsp. mellifera
23. B. asteroides	16	H	Intestine of A. mellifera L. subspp. mellifera, ligustica and caucasica; (A. cerana F.)
24. B. indicum	16	H	Intestine of A. cerana F. and A. dorsata F.

[a] Taken from Scardovi et al., 1971a; F6PPK = fructose-6-phosphate phosphoketolase. Numbers indicate the migration relative to that of B. globosum taken = 10. Phosphoketolases of migration 15 and those of migration 10 were ecologically distinguished as "human" and "animal" type, respectively (Sgorbati et al., 1976).

[b] Taken from Sgorbati and London, 1982.

[c] Sources are listed in the order of decreasing frequency of occurrence of the species therein. Sources in parentheses are occasional.

Bifidobacteria in Domestic Animals

The composition of the fecal flora of healthy adult animals was determined by Mitsuoka (1982) and the results are presented in the table below.

Fecal flora of twelve adult animals of different species

Bacterial groups	Monkeys	Chickens	Pigs	Dogs	Cats	Minks
Total counts	10.7±0.4[a](5)[b]	10.9±0.2 (5)	10.8±0.4 (5)	10.8±0.2 (5)	10.2±0.2 (5)	9.8±0.2 (5)
Bacteroidaceae	10.1±0.4 (5)	10.6±0.2 (5)	10.3±0.8 (5)	10.3±0.3 (5)	9.7±0.4 (5)	7.6±1.5 (5)
Eubacteria	10.0±0.6 (5)	10.2±0.3 (5)	9.2±1.0 (5)	9.9±0.4 (5)	9.4±0.5 (5)	8.4±0.1 (5)
Peptococcaceae	9.8±0.4 (5)	9.9±0.1 (5)	9.8±0.3 (5)	9.6±0.5 (5)	9.6±0.1 (5)	0 (0)
Anaerobic curved rods	9.4±0.2 (2)	0 (0)	9.4±0.3 (5)	8.8±0.7 (2)	9.0 (1)	0 (0)
Bifidobacteria	9.8±0.5 (5)	9.1±0.9 (5)	9.0±0.5 (5)	9.2±0.8 (5)	0 (0)	0 (0)
Lactobacilli	8.9±0.7 (5)	9.5±0.5 (5)	9.9±0.4 (5)	9.6±0.6 (5)	5.2±1.5 (4)	6.1±0.1 (5)
Veillonellae	5.5±1.9 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Clostridia	0 (0)	0 (0)	6.9±1.0 (4)	5.6±1.5 (3)	9.2±0.4 (5)	7.4±1.1 (5)
Fusiform bacteria	0 (0)	0 (0)	0 (0)	9.0±0.7 (2)	0 (0)	0 (0)
Spirochaetes	10.2 (1)	0 (0)	9.5±0.8 (3)	0 (0)	7.3 (1)	0 (0)
Enterobacteriaceae	7.2±1.0 (5)	7.0±0.4 (5)	8.1±0.1 (5)	8.1±1.0 (5)	7.9±0.4 (5)	9.6±0.1 (5)
Streptococci	7.3±1.4 (5)	7.1±0.4 (5)	7.9±1.0 (5)	9.9±0.4 (5)	8.5±0.4 (5)	9.2±0.3 (5)
Staphylococci	4.2±0.5 (5)	6.8±0.7 (5)	3.5±1.1 (3)	3.4±0.8 (4)	6.8 (1)	5.7±0.8 (4)
Corynebacteria	0 (0)	8.6 (2)	6.5±0.5 (2)	0 (0)	0 (0)	0 (0)
Bacilli	6.6 (1)	6.4±1.2 (5)	6.4±0.9 (5)	0 (0)	0 (0)	0 (0)
Yeasts	4.5±1.4 (5)	4.2±1.1 (5)	4.2±0.1 (2)	3.4±0.7 (4)	3.4±1.6 (2)	5.7±0.4 (5)

[a] Mean±SD of log bacterial counts (when present).
[b] Figures in parentheses refer to the number of subjects that harbor the organism.

Table (Continued)

Bacterial groups	Mice	Rats	Hamsters	Guinea pigs	Rabbits	Horses
Total counts	10.7±0.3 (5)	10.4±0.2 (5)	10.3±0.2 (5)	9.5±0.2 (5)	9.7±0.2 (5)	9.0±0.4 (5)
Bacteroidaceae	10.5±0.3 (5)	9.9±0.2 (5)	9.9±0.4 (5)	8.5±0.7 (5)	9.6±0.2 (5)	7.2±1.6 (5)
Eubacteria	9.1±0.7 (5)	9.5±0.3 (5)	0 (0)	8.1±0.4 (5)	5.6±1.0 (2)	7.7±0.3 (3)
Peptococcaceae	8.9±0.2 (2)	9.3±0.3 (5)	9.7±0.2 (5)	9.1±0.3 (5)	8.3±1.0 (5)	6.8±2.4 (5)
Anaerobic curved rods	8.9±0.5 (3)	9.5±0.4 (5)	9.2±0.5 (5)	8.7 (1)	8.6±0.3 (5)	8.3±0.4 (5)
Bifidobacteria	7.1±1.2 (4)	8.2±0.8 (5)	9.0±0.3 (5)	8.8±0.3 (5)	7.8 (1)	8.5±0.8 (5)
Lactobacilli	9.5±0.4 (5)	9.6±0.3 (5)	9.7±1.2 (5)	8.2±0.7 (5)	0 (0)	7.7±0.5 (5)
Veillonellae	0	4.5±0.3 (5)	4.5±0.5 (5)	2.6±0.3 (3)	0 (0)	4.6±0.4 (5)
Clostridia	8.6±0.5 (4)	2.1 (1)	0	0	2.3 (2)	7.5±0.4 (5)
Fusiform bacteria	9.8±0.5 (5)	9.2±0.5 (5)	9.6±0.7 (5)	0 (0)	0 (0)	0 (0)
Spirochaetes	7.5±0 (1)	0 (0)	0 (0)	0 (0)	0 (0)	7.6±0.5 (5)
Enterobacteriaceae	4.7±1.2 (5)	5.3±1.4 (5)	6.3±0.7 (5)	6.4±1.6 (5)	3.5±1.3 (4)	5.5±1.0 (5)
Streptococci	5.6±0.9 (5)	8.2±0.6 (5)	5.1±1.5 (5)	6.9±1.8 (5)	3.6±0.6 (3)	8.5±0.8 (5)
Staphylococci	4.8±0.7 (5)	5.8±1.3 (5)	4.8±0.6 (5)	7.3±0.7 (2)	3.4 (1)	3.8±0.6 (5)
Corynebacteria	0 (0)	0 (0)	0 (0)	8.3±0.2 (4)	4.6±0.4 (2)	3.9±0.4 (2)
Bacilli	3.5±0.4 (4)	0 (0)	4.4 (1)	7.9±0.4 (5)	0 (0)	6.1±1.0 (5)
Yeasts	0 (0)	0 (0)	0 (0)	2.4 (1)	4.3 (1)	2.8±0.2 (4)

In general, animals of the same species had a common pattern of fecal flora but the patterns differed from those of other species. The most predominant fecal bacteria in almost all animal species were the anaerobes bacteroidaceae, bifidobacteria, eubacteria, lactobacilli, peptococcaceae, and anaerobic curved rods. Lactobacilli and bifidobacteria numbers were found to vary with the species of animal.

Resnick and Levin (1981) examined the feces of humans, cows, chickens, dogs, pigs, horses, cats, sheep, beavers, goats and turkeys but bifidobacteria were isolated only from the feces of humans and swine on YN-6 medium. The species found in the feces of adult swine were B. thermophilum, B. longum, B. pseudolongum, and B. adolescentis.

In contrast Beerens (1991) using a selective medium containing propionic acid was able to recover bifidobacteria from the feces of the domestic rabbit, horse, hen, swallow, crow, domestic rat, dog, pig, fly and fox.

Bifidobacteria in Water, Sediment and Sewage

There have been only a limited number of studies in which attempts have been made to quantitate bifidobacteria. Evison and James (1973), working in the United Kingdom and Kenya, have shown that the densities in water samples were generally comparable to the E. coli levels of approximately 10^2/100 mL. In sewage there was a ten fold higher concentration of bifidobacteria relative to E. coli densities (10^6/100 mL versus 10^5/100 mL).

Carrillo and co-workers (1985) measured the density of Bifidobacterium spp., fecal coliforms, E. coli, and total anaerobic bacteria, acridine orange direct counts, percentages of total bacterial community activity and respiration, and 12 physical and chemical parameters in the Mameyes River rain forest watershed. The watershed has a total length of 17 km and average precipitation in the upper third of the watershed is 395 cm. The middle third of the watershed has agricultural land and several housing projects that dump their sewage into the River. The lower third is dominated by two towns that contribute wastes from municipal, domestic, and light industry to the river.

As shown in the Table below the highest densities of fecal coliforms, E. coli, and Bifidobacterium spp. were found at site 9, the sewage point source. However, site 1 had significantly higher densities of each of these bacteria than the sites below it, with the exception of site 9. Site 1 also had the highest density of total anaerobes of any of the sites.

Site No.	Mean Density[a] of Fecal coliforms	E. coli	Bifidobacteria
1	1.3×10^2	1.0×10^2	5.6×10^2
3	0.7×10^2	0.3×10^2	3.4×10^2
4	0.2×10^2	0.1×10^2	0.3×10^2
5	0.1×10^2	0.1×10^2	0.6×10^2
7	1.9×10^2	0.4×10^2	1.3×10^2
9	184.0×10^2	134.0×10^2	6.5×10^2

[a] All units are in CFU per 100 mL.

Densities of fecal coliforms reported in the Carrillo et al. study were lower than those reported by Evison and James (1975) for river samples taken in tropical Africa and much higher than those reported for river samples taken in England. Site 9, which received sewage effluent, had densities of fecal coliforms and E. coli that were 2 orders of magnitude higher than uncontaminated sites.

In situ diffusion chamber studies demonstrated that E. coli could survive indefinitely in the rain forest watershed. Regrowth of E. coli was also evident.

Daily and coworkers (1981) studied the seasonal occurrence of obligate anaerobes at a diver training site on the Anacostia River in Washington, D.C. The depth of the water at the sampling site was 6.1 m. Water samples were collected 1m below the surface and 1m above the bottom; sediment samples were collected with a grab sampler. The results showed that the total anaerobic counts varied from 1.8×10^3 cells/mL in the warmer months to 10 cells/mL in the winter. One isolate of each of B. animalis, B. breve, and B. longum was recovered from the bottom water and one isolate of B. magnum was obtained from the top water.

Bifidobacteria as Indicators of Fecal Contamination

Mossel is credited with initially proposing the use of the bifidobacteria as indicators in 1958 (D.A.A. Mossel, Abstr. 7th Int. Congr. Microbiol., 1958, p. 440): however, the significance of these organisms in sanitary microbiology is still unclear.

A great deal of work is required to develop the techniques necessary to determine the true potential of bifidobacteria as indicators of fecal pollution. In theory, the genus has all of the characteristics of an excellent, if not ideal, indicator: a) the organisms do not multiply in nature; b) the sole source is fecal material; c) its survival characteristics make bifidobacteria an indicator of recent pollution; and d) the possibility of developing a reliable test to determine the source of pollution (human or animal) exists.

Determination of Ratios of Indicator Organisms

The density of Bifidobacterium spp., fecal coliforms, E. coli, and total anerobic bacteria was determined by Carrillo et al. (1985) for 12 months in the Mameyes River rain forest watershed, Puerto Rico. Their previous studies showed that temperature is constant throughout the year and this watershed exhibits no seasonal differences in water quality (Hazen and Aranda, 1981). The densities of organisms found in the watershed are provided in the "Bifidobacteria in Water, Sediment and Sewage" section. The results, presented below for each site, showed that the ratio of bifidobacteria to E. coli and to fecal coliform bacteria was significantly lower at site 9, the sewage point source, than any of the other sites.

	Ratios	
Site no.	Bifidobacteria/fecal coliforms	Bifidobacteria/E. coli
1	11.3	16.6
3	4.2	22.7
4	1.8	3.9
5	3.5	4.0
7	2.1	17.1
9	0.2	0.4

For comparative purposes, ratios of mean densities of fecal coliforms and E. coli to bifidobacteria were calculated, as shown below. Similar conclusions could be drawn, i.e. the sewage source site produced a markedly different ratio compared with those observed at the other sites.

	Ratios of mean densities of	
Site no.	Fecal coliforms/bifidobacteria	E. coli/bifidobacteria
1	0.2	0.2
3	0.2	0.1
4	0.7	0.3
5	0.2	0.2
7	1.5	0.3
9	28.3	20.6

Further work in this area of indicator ratios is warranted.

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PART I

BACTERIAL INDICATORS FOR THE DETECTION OF SANITARY WASTE IN STORM SEWERS

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ABSTRACT

Storm sewers are designed to channel untreated storm water into surface waters. An unusually high dry weather flow of storm sewage along with high fecal coliform counts indicate that there may be potentially hazardous sanitary connections in the storm sewer line. The objective of this study was to assess the use of bacterial indicators to trace illegal sanitary connections to storm sewers. The indicators selected for study were Escherichia coli, fecal coliforms, fecal streptococci, enterococci, Pseudomonas aeruginosa, Clostridium perfringens, and Bifidobacterium sp. The organisms were collected from specific sites in sanitary sewer lines, and from priority and nonpriority storm sewers during periods of wet and dry weather as well as from surface water runoff. The results showed that Streptococcus casseliflavus was primarily recovered from surface runoff and nonpriority storm sewers. On the other hand, bifidobacteria concentrations were higher in sanitary and high priority storm sewage than in nonpriority storm water. These data suggest that a ratio of Bifidobacterium to S. casseliflavus counts may be used to trace the location of human and/or animal fecal wastes. Additional field studies are needed to confirm the applicability of these ratios.

KEYWORDS

Storm sewers; sanitary waste; bifidobacteria; Streptococcus faecium subsp. casseliflavus; fecal indicators.

INTRODUCTION

The Ministry of the Environment has designated storm sewer outfalls discharging more than 1 L/sec. during dry weather periods and exhibiting fecal coliform densities of greater than 10,000 FC/100 mL as high priority. Efforts were made to identify and eliminate the source of the fecal pollution in these sewage lines but pinpointing the original source of pollution has been difficult because specific bacterial indicators of human fecal waste were not available.

Traditionally, Escherichia coli and fecal coliforms have been used as the indicators of fecal pollution in storm waters and surface waters but, due to their wide distribution in both human and animal feces, they are not acceptable as an indicator of strictly human input.

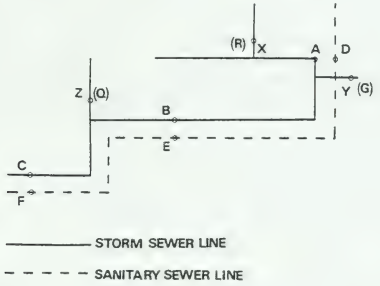
Mossel (1958) was the first to propose bifidobacteria as potential indicators of fecal contamination. More recently, a number of authors (Cabelli, 1979; Resnick and Levin, 1981) have suggested that bifidobacteria could be used to distinguish between human and animal fecal pollution. However, the usefulness of bifidobacteria as a specific indicator of human fecal pollution may be limited due to the short survival of the organism in the extra-intestinal environment (Resnick and Levin, 1981; Oragui, 1982).

The purpose of this study was to characterize the bacterial populations found in urban storm and sanitary wastes in an attempt to develop a methodology for the detection of human fecal wastes in storm sewer lines.

METHODS

Sampling Sites. Figure 1 indicates the sites surveyed in the Mount Steven Trunk storm sewer line. Locations A, B and C were sampled because this area was designated a high priority sewer by the Ministry of the Environment. The nonpriority sites, selected for comparison, were X, Y and Z in the Mount Steven Trunk storm sewer branch lines. During periods of wet weather, storm water run-off was also collected at the X, Y and Z sites. These samples were labelled R, G and Q, respectively. Samples D, E and F were obtained from a sanitary sewer in close proximity to the priority storm sewer sampling points.

Fig. 1. Schematic diagram of Mount Steven storm sewer and sanitary sewer lines showing sampling locations



Sample Collection. During periods of dry weather, triplicate samples were collected from each sampling point in the sewers over a four-day period. The dry weather samples were obtained in October, 1986; June, 1987; August, 1987; June, 1988; and August, 1988. Wet weather samples were collected from the sewers and the street run-off during rainy days in July, September and October, 1987.

The samples were collected in sterile glass containers, transported to the laboratory on ice, and processed within 6 hours of collection.

Fecal specimens from both humans and animals were also obtained.

Bacterial Isolation and Enumeration. Analysis of the samples for indicator bacteria was by membrane filtration of appropriate dilutions of the samples through Gelman GN6 47 mm cellulose nitrate filters with a pore size of 0.45 um (APHA, 1985). The filters were planted on media appropriate for the recovery of the various indicator bacteria. The upper and lower counting limits of target organisms on each of the media were noted.

Fecal coliform bacterial densities were determined by planting the filters on m-TEC agar (Dufour et al. 1981) and incubating for 23 $\pm$ 1 hours at 44.5 $\pm$ 0.5° C. Both target and non-target colonies were counted. Target colonies were yellow, yellow-green and yellow-brown; non-target colonies were blue to blue-green in colour.

A second step for the determination of E. coli by urease treatment (Dufour and Cabelli, 1975) was incorporated into the m-TEC procedure. Filters with appropriate target counts (i.e. between 10 and 100 target colonies) were removed from the m-TEC plates and placed on filter pads soaked in a urea phenol red solution (Dufour and Cabelli, 1975). The filters remained in contact with the filter pad for 15 min to allow for deaminization by non-E. coli coliform bacteria

processing urease. A second count of all urease negative colonies (all yellow, yellow-green and yellow-brown colonies) was taken.
Fecal streptococci determinations were made by planting the filters on m-Enterococcus agar (Difco) (APHA, 1985). The medium was incubated for 48 hr at 35° C and a count of all pink to purple target colonies taken.

Enterococci were recovered on m-ME agar (APHA, 1985) which also contains indoxyl-β-D-glucoside (IG). The m-ME plates were incubated for 48 hr at 41.5 $\pm$ 0.5° C. The incubation time was modified from the original 24 hr suggested by Dufour as it allowed for a slight increase in recovery of target organisms. The addition of IG to the medium facilitates differentiation of the β-D-glucosidase enterococci from other fecal streptococci. Both target and non-target colonies were enumerated. Target colonies on m-ME were purple, white-blue to dark blue with blue haloes from degradation of the IG. Non-targets were pink to maroon non-haloed colonies.

Pseudomonas aeruginosa densities were determined using m-PA agar (APHA, 1985). The medium was incubated at 41.5 $\pm$ 0.5° C for 48 hr and a count of all flat spreading brownish-green or tan colonies obtained.

Bifidobacterium spp. were isolated on the YN-17 medium described by Mara and Oragui (1983). Target bifidobacteria colonies appear dark blue to black. Greenish coloured colonies were not counted; however, to ensure that all bifidobacteria

were enumerated, crystal violet stains were made of colonies that had a different size or colour. Adjustments to the counts were made if necessary.

Clostridium perfringens was isolated using a medium originally developed by Bisson and Cabelli (1979) and modified by the Ministry of the Environment Southeastern Region Laboratory (m-CP2). The samples were pretreated at 70° C for 15 or 30 min to destroy the vegetative cells. Appropriate volumes of the samples were passed through membrane filters and the filters were placed on m-CP2 plates. The plates were then incubated anaerobically at 37° C for 48 hr. Target colonies appear yellow with a large black centre which can extend fairly close to the circumference; the colonies do not possess a blue halo. Non-target colonies have a blue halo and they can be yellow or yellow with a black centre.

All bacteria were identified to the species level using standard taxonomic methods.

Bifidobacteria and E. coli Survival Studies. The 50 mL dialysis membrane diffusion chambers used in the study were designed by McFeters and Stuart (1972). The 48 hr broth cultures of E. coli, B. breve, and B. bifidum as well as the fecal and sewage isolates of bifidobacteria were centrifuged and washed three times in gelatin phosphate buffer. Following resuspension and dilution, the bacteria were suspended in sterile L. Ontario water and added to the chambers. The chambers were then immersed in a glass tank containing L. Ontario water at room temperature. Disposable 10 mL luer-lock syringes were used to collect the bacterial samples at 2, 5, 12, 24, 48 and 72 hr. Bacterial densities were enumerated by appropriately diluting, membrane filtering and planting the filters on YN-17 and m-TEC media. All samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Enumeration of Fecal Indicator Bacteria in Sewers. Levels of fecal coliforms (FC), Escherichia coli (EC), fecal streptococci (FS) and Pseudomonas aeruginosa were monitored in storm and sanitary sewage during dry weather in the fall of 1986. The three sites (A, B and C) in the high priority storm sewer had FC geometric mean concentrations ranging from 10^3 to 10^4 with an EC range of 10^2 to 10^3. It should be noted that FC and EC concentrations of this magnitude are suspiciously high for storm water and suggest an input of sanitary waste contamination. The densities of FS were in a slightly lower range and P. aeruginosa levels were low in comparison with the other bacterial indicators.

As would be expected, the FC, EC and FS concentrations in sanitary sewage were approximately an order of magnitude higher than the levels observed in storm sewers. The P. aeruginosa count was observed to be around 10^4 per 100 mL in the sanitary sewers; this was considerably higher than the 10^1 counts observed in storm sewers.

During a dry weather survey the following year (Figs. 2 and 3), comparable results were obtained for FC and EC densities. As may be seen in Fig. 2 the observed counts were higher at points A and B, the suspected site of contamination in the storm sewer. Analysis of Bifidobacterium and Clostridium perfringens levels in storm and sanitary sewage produced interesting results. As was observed for the FC and EC determinations the bifidobacteria counts were highest at sites A and B in the storm sewer and dropped off at point C. Clostridium perfringens levels, on the other hand, increased rather than decreased at point C. This might be expected since, rather than dying off, C. perfringens spores have a tendency to build up and accumulate in the system. Because C. perfringens is present in high densities in human and animal feces, levels in the sanitary sewage were in the 10^4 range compared with 10^2 densities in storm sewage.

The aforementioned enumeration results show that levels of all the bacterial indicators demonstrate that there is fecal input in the storm sewer line. Bifidobacteria and P. aeruginosa appear to have potential as indicators of a human source of contamination. Because C. perfringens produces spores that remain in the system, this organism is not effective as a point-of-impact indicator.

Bifidobacteria were isolated from fecal specimens in concentrations 10 to 100 times greater than E. coli. However, in sewage samples the counts of Bifidobacterium were lower than those of E. coli. This could be due to a dilution effect and also to the fact that species of Bifidobacterium tend to die off at a faster rate in the environment (Oragui, 1982).

Speciation of Bifidobacteria. When the Bifidobacterium isolates were speciated it was found that a wider variety of bifidobacteria species were recovered from sewage samples than from fecal samples (Fig. 4). The predominant species in human feces and sewage were the sorbitol-fermenting B. adolescentis and B. breve. As indicated in Fig. 4 B. breve was isolated from cat fecal samples but none of the cat isolates were found to be sorbitol-fermenting. Mara and Oragui (1983) have proposed that sorbitol-fermenting bifidobacteria be used as indicators of human fecal pollution. Because, as cited earlier (Seyfried et al. 1988), we

have been able to isolate B. adolescentis from dogs and B. breve from chicken feces this approach should be viewed with caution.

Bifidobacterium Survival. The advantages of using bifidobacteria as fecal indicator bacteria are that they exist in high numbers in both feces and fecally contaminated environments. Furthermore, they supposedly do not multiply outside the body. The limitations associated with the use of bifidobacteria as a specific indicator of human fecal pollution relate to the organism's short survival time outside the intestine (Resnick and Levin, 1981; Oragui, 1982). Previous survival studies have shown bifidobacteria to die off more rapidly than E. coli upon exposure to environmental waters (Carillo, Estrada and Hazen, 1985). Because too rapid a decline of bifidobacteria in the aquatic environment and subsequent indetectability could make the organism a poor indicator of fecal contamination this study compared the in vitro survival characteristics of bifidobacteria and E. coli using the dialysis membrane filter chambers devised by McFeters and Stuart (1972). Pure cultures of B. breve and B. bifidum as well as fecal and sewage isolates of bifidobacteria were studied. The comparative survival rates of B. breve and E. coli in Lake Ontario water at temperatures of 21 to 23° C are depicted in Fig. 5. In all cases the bifidobacteria cultures appeared to parallel the die-off trends in E. coli during the initial five hour period; thereafter, bifidobacteria began to show a slightly more rapid decline. The Bifidobacterium isolates died off within 24 hr of exposure to L. Ontario water whereas E. coli persisted for 48 hr. Dilution of nutrients and exposure of the pure culture to any toxic chemicals present in the water may have had an impact on the survival of the organisms.

The more rapid decline of the anaerobic bifidobacteria in comparison with the aerobic E. coli may be accounted for by oxygen diffusion into the chamber. The propensity of the bifidobacteria to be present in feces and to die off rapidly in the environment make them good indicators of recent fecal pollution.

Speciation of the fecal streptococci provided more insight into the origin of fecal wastes in storm sewer lines. The results showed that S. faecium tended to be equally represented in all sample categories. On the other hand, S. faecalis subsp. faecalis (Fig. 6) was found more frequently in sanitary and priority storm sewers than in surface runoff and non-priority sewers. In contrast, S. faecium subsp. casseliflavus (Fig. 7) predominated in non-priority storm sewer water and was notably evident in storm water runoff. The organism was virtually nonexistant in sanitary sewage and levels in priority storm sewers were small.

Field studies are currently underway to assess the feasibility of using fecal coliform: S. faecium subsp. casseliflavus or bifidobacteria: S. faecium subsp. casseliflavus ratios to distinguish human from animal sources of pollution.

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Fig. 2 Geometric mean concentrations of fecal coliforms, E. coli, Bifidobacterium spp., and Clostridium perfringens in storm sewage during the June 1987 dry weather survey.

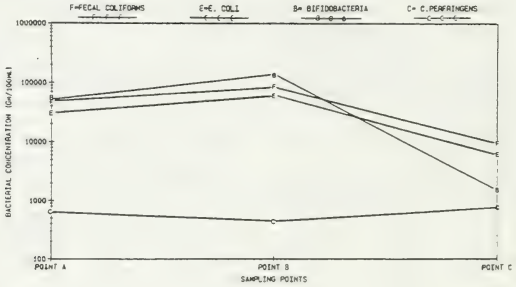

Fig. 3 Geometric mean concentrations of fecal coliforms, E. coli, Bifidobacterium spp., and Clostridium perfringens in sanitary sewage during the June 1987 dry weather survey.

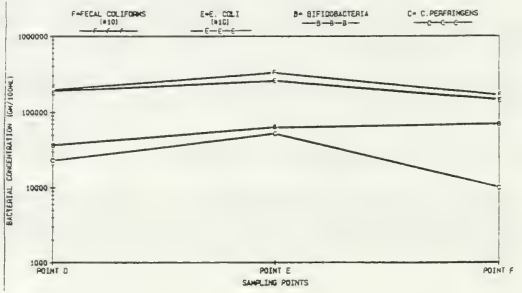

Fig. 4 Percent distribution of bifidobacteria in feces, sanitary and storm sewage.

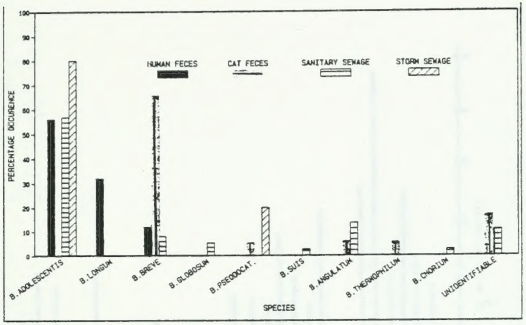

Fig. 5 In-vitro survival of Bifidobacterium breve and E. coli in Lake Ontario water.

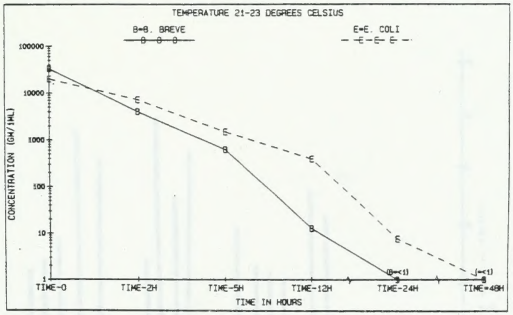

Fig. 6 Percent distribution of Streptococcus faecalis subsp. faecalis among the sanitary sewage sites (D, E, F) the priority storm sewer (A, B, C, Y), the non-priority storm sewer (X, Z) and the storm water runoff (P, G, R, Q) locations.

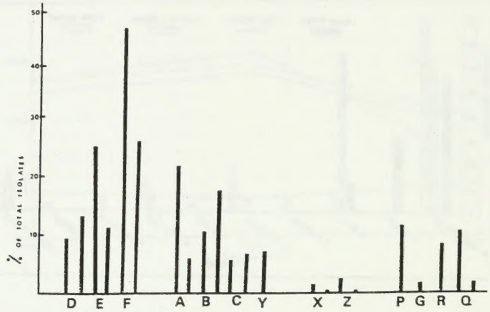

Fig. 7 Percent distribution of Streptococcus faecium subsp. casseliflavus among the sanitary sewage sites (D, E, F), the priority storm sewer (A, B, C, Y), the non-priority storm sewer (X, Z) and the storm water runoff (P, G, R, Q) locations.